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## 13. ABSTRACT (Maximum 200 words)

Information is needed on mechanisms by which humans respond to exposure of their cellular genes to toxic chemicals in the environment. The principal DNA repair mechanism in organisms is excision repair and evidence has accumulated that these mechanisms are highly conserved. Using the fruit fly as a eukaryotic model, we undertook the molecular cloning of excision repair genes on the basis of their potential structural and functional similarity to the well-characterized excision repair genes in yeast.

cDNA libraries were constructed from mRNA isolated from Drosophila embryos in a yeast expression vector, pYES 2.0, and subsequently used to rescue a yeast rad3 mutant strain, known to be defective in excision repair. Although the equivalent of four genome equivalents were screened for complementation, no functional cognate Drosophila gene was recovered.

On the basis of conserved DNA sequence between the yeast RAD3 and the human ERCC-2 genes, the polymerase chain reaction was used to recover a Drosophila cognate sequence. Using our cDNA library as template, a single DNA band was identified. We interpret this to mean that Drosophila does have a RAD3 cognate but it was not represented in a functional form in our cDNA library to allow rescue of the rad3 mutant strain.

## 17. SECURITY CLASSIFICATION OF REPORT

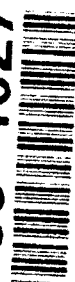
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## 18. SECURITY CLASSIFICATION OF THIS PAGE

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## List of Objectives

Research supported by the Air Force Office of Scientific Research seed grant, AFOSR-90-0289, was undertaken to develop a system which would allow us to expand our understanding of cellular mechanisms of DNA repair in eukaryotes. Experiments were designed to accomplish three objectives:

1. Construction of a *Drosophila melanogaster* cDNA library in the *Saccharomyces cerevisiae* expression vector, pYES2.0, starting with poly(A<sup>+</sup>) RNA from DNA excision repair-proficient Oregon-R strain embryos;
2. Development of a protocol for the employment of the cDNA expression library to rescue mutagen-sensitive, DNA excision repair-deficient (*rad*) yeast cells from killing by treatment with mutagenic chemical and physical agents; and,
3. Development of a polymerase chain reaction assay to isolate *Drosophila* DNA sequences, expected to be central to the DNA excision repair process, based on homology to sequences from other organisms shown to be strongly conserved between widely divergent species.

## Accomplishments

Accomplishments for this seed project will be discussed under two headings:

- I. *Molecular Cloning by Functional Complementation*
- II. *Molecular Cloning by PCR-based Sequence Homology.*

### I. *Molecular Cloning by Functional Complementation*

Our initial approach focussed on the identification of a *Drosophila* gene which would have functional similarity to the nucleotide excision repair gene, RAD3, of *Saccharomyces*. RAD3, the prototypical excision repair gene, after which the excision repair pathway of yeast is named, provides an ideal gene for the development of these techniques. The human excision repair gene, ERCC-2, has over 50% conservation to the yeast RAD3 gene (Weber et al., 1990), suggesting that this gene has remained highly conserved during evolution and was likely to have a *Drosophila* cognate.

The mutant *rad3* strain of choice for the proposed complementation experiment, LP2649 (MAT alpha, *rad3-2 leu2-3,112 ura 3-52 can1-r*), was analyzed for both survival after treatment with mutagenic agents and vector transformability. The use of a *rad3* hypomorphic mutant with minimal survival capability and the

definition of the lowest level of mutagenic treatment which kills this cell in the absence of *any* significant excision repair function was important.

Table 1 shows the necessary *minimal* level of gene function that would be needed to distinguish rescue of repair-proficient transformants from untransformed mutant cells. Repair-proficient background is insignificant and 1% wild type function can readily be screened. This particular rad3 allele was an excellent choice for these experiments.

Table 1. Comparison of the UV sensitivities of rad3-2 and rad3-2 with the RAD3<sup>+</sup> gene inserted into a LEU2<sup>+</sup> selectable transformation vector

<u>STRAIN</u>	<u>UV DOSE</u>	<u>TITER</u>	<u>% SURVIVAL</u>
CG1241 (rad3-2)	0 J/m <sup>2</sup>	1.56 × 10 <sup>7</sup> CFU/ ml.	100 %
	5 J/m <sup>2</sup>	1.73 × 10 <sup>6</sup> CFU/ ml.	11.1 %
	10 J/m <sup>2</sup>	5.40 × 10 <sup>4</sup> CFU/ ml.	0.35 %
	20 J/m <sup>2</sup>	1.5 × 10 <sup>2</sup> CFU/ ml.	0.00096 %
	30 J/m <sup>2</sup>	8.5 × 10 <sup>1</sup> CFU/ ml.	0.00054 %
	40 J/m <sup>2</sup>	1.5 × 10 <sup>1</sup> CFU/ ml.	0.000096 % (background 0)
CG1240 (rad3-2/RAD3 <sup>+</sup> )	0 J/m <sup>2</sup>	9.75 × 10 <sup>6</sup> CFU/ ml	100 %
	20 J/m <sup>2</sup>	4.54 × 10 <sup>6</sup> CFU/ ml	46.6 %
	50 J/m <sup>2</sup>	3.11 × 10 <sup>6</sup> CFU/ ml	31.9 %
	100 J/m <sup>2</sup>	1.02 × 10 <sup>6</sup> CFU/ ml	10.5 %
	50 J/m <sup>2</sup>	4.3 × 10 <sup>5</sup> CFU/ ml	4.46 %
	250 J/m <sup>2</sup>	3.95 × 10 <sup>3</sup> CFU/ ml	0.04 %

Protocol:

1. 50 ml. cultures of yeast strains were grown in leucine minus dropout medium (synthetic medium supplemented with the other requirements of the strain, but lacking leucine), to force maintenance of the LEU2<sup>+</sup> plasmids, at 30° with rapid aeration. Growth was monitored until late exponential phase was reached (titer of ~ 1 - 2 × 10<sup>7</sup> cells/ ml.).
2. Cultures were harvested by centrifugation and washed 3 times with 50 ml. of sterile water, to remove UV absorbing media components. Cells were resuspended at a titer of 1 - 2 × 10<sup>7</sup> cells/ ml. in sterile water (~ 40 ml.).
3. Sequential ten fold dilutions of the washed cultures were performed in sterile water and plated by spreading onto leucine minus plates, to force maintenance of the LEU2<sup>+</sup> plasmids.
4. Plated cultures were exposed to 254 nm UV irradiation from a UV Products germicidal lamp (Model UVGL-25, 4 watts), which was calibrated to deliver at 1.0 J/m<sup>2</sup>-sec of 254 nm UV radiation using a UVX digital radiometer with a 254 nm sensor. Irradiation was performed in a darkened room and plates were immediately placed into a light proof box after exposure to UV, in order to prevent photoreactivation.

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5. Plates were incubated at 30° for 3 days and then the viable count was determined as CFU/ ml. (colony forming units per milliliter of irradiated culture).

Another experimental parameter which is important for *maximizing* the ability to identify a strain which could withstand mutagenic treatment with *minimal* interspecific gene expression is the maximization of transformability of the mutant rad strain with the specific vector to be used for the rescue attempt. Table 2 reports the results of transformation experiments in which the pYES2.0 and pGB620 vectors were used to optimize transformation frequency by electroporation.

Table 2. Efficient transformation by electroporation of rad3-2.

<u>DNA</u>	<u>CARRIER DNA</u>	<u>URA3 TRANSFORMANTS</u>
none	4 µg ds salmon sperm	$2 \times 10^1$ / ml. (background, control)
0.1 µg pGB620	none	$8.2 \times 10^2$ / ml. (410 x above bkg.)
0.1 µg pGB620	4 µg ds salmon sperm	$2.99 \times 10^4$ / ml. (1500 x above bkg.)
1.0 µg pGB620	40 µg ds salmon sperm	$1.33 \times 10^4$ / ml. (665 x above bkg.)
1.0 µg pGB620	4 µg ds salmon sperm	$4.14 \times 10^4$ / ml. (2070 x above bkg.)
0.1 µg pYES 2.0	4 µg ds salmon sperm	$7.8 \times 10^3$ / ml. (390 x above bkg.)
1.0 µg pYES 2.0	4 µg ds salmon sperm	$2.88 \times 10^4$ / ml. (1440 x above bkg.)

#### PROTOCOL

1. A 250 ml. culture of recipient strain LP2649 (rad 3-2) was grown overnight in YPDA (rich medium) at 30° until it reached a titer of  $1.14 \times 10^8$  cells/ ml. (late exponential growth phase).
2. The culture was harvested by centrifugation at 4° and resuspended in 50 ml. of pretreatment buffer (20mM Hepes pH8, 25mM DTT). (5x concentration,  $\sim 5 \times 10^8$  cells/ ml.)
3. Cells were pretreated at 30° for 30 minutes with gentle shaking.
4. Cells were washed twice by centrifugation, with 250 ml. of ice cold sterile water.
5. Cells were gently resuspended in 10 ml. ice cold sterile 1M sorbitol (25 x concentration,  $\sim 2.5 \times 10^9$  cells/ ml.) and pelleted by centrifugation.
6. The cell pellet was resuspended by mixing with 0.5 ml. of ice cold sterile 1M sorbitol; the final volume was approximately 1.8 ml. ( $\sim 1.58 \times 10^{10}$  cells/ ml.).
7. 100 µl of cell suspension ( $\sim 1.58 \times 10^9$  cells) was put in a chilled 0.2 cm. Bio-Rad electroporation cuvette. Transforming DNA and carrier DNA was added as described below, in a total volume of 10 µl or less.
8. Electroporation was performed in the Bio-Rad Gene Pulser with a pulse setting of 1.5 kv, 200 Ω and 25 µF (pulse time constant of 4.0 - 4.6 msec.).
9. Electroporated cells were diluted in sterile 1M sorbitol and plate on uracil minus dropout plates (synthetic medium supplemented with the other strain requirements but lacking uracil) to select for URA3<sup>+</sup> transformants. Plates were incubated for 2 days at 30° to allow growth of transformants.

These toxicology and transformation experiments suggested that we would be able to select for rare transformants expressing very modest levels of interspecific

excision repair ability and be able to distinguish these cells from the original repair-deficient mutants.

Studies were then conducted to gain experience with the construction of cDNA libraries from *Drosophila melanogaster* wild-type Oregon-R strain embryos. These studies were particularly important because (1) we know that excision repair gene expression is present in embryonic cells (Dusenbery et al., 1983; Dusenbery, 1987) but we anticipate that this expression will be at low levels and (2) we had no prior experience with RNA work. Table 3 outlines the experimental protocol which we followed for these cDNA constructions.

Table 3. Protocol (and Controls) for cDNA library constructions

- A. Preparation of embryos
  1. Collect 0-12 hr Oregon-R embryos on grape plates in population cages.
  2. Wash repeatedly over 100m stainless steel screens with diethylpyrocarbonate (DEPC)-treated deionized H<sub>2</sub>O.
  3. Freeze in liquid N<sub>2</sub> and store at -80°C in cryovials at 1gm/vial until needed for experimental procedures.
- B. Preparation of polyA<sup>+</sup> RNA
  4. Homogenize frozen embryos in 4M guanidinium isothiocyanate and precipitate RNA with ETOH. Remove contaminating proteins with repeated ETOH precipitation from guanidine HCl.
  5. Passage RNA 2X through oligo-dT columns (see Figure 2).
  6. Check quality and purity (no ribosomal RNA contamination) of polyA<sup>+</sup> RNA by alkaline agarose gel electrophoresis (see Figure 2).
  7. Determine intactness of polyA<sup>+</sup> RNA by Northern analysis for representative messages from oligo-dT column fractions (actin, *ADH*, *ry*) (data not shown).
- C. Preparation of cDNA library
  8. Using 5mg of polyA<sup>+</sup> RNA from individual or pooled oligo-dT column fractions primed with oligo-dT, first-strand synthesis is conducted with Gibco/BRL Superscript reverse transcriptase. Second strand synthesis is immediately performed with the addition of dNTPs, RNase H, and Klenow fragment of *E. coli* DNA polymerase. To insure blunt-ended molecules, there is a brief treatment with T4 DNA polymerase. First-strand synthesis is monitored radioactively with a control sample and routinely gives a highly acceptable incorporation rate of approximately 30%.
  9. Following phenol/chloroform extraction and ETOH precipitation, BstX1/EcoR1 adapters are ligated to cDNAs for cloning in pYES2.0 and ligation products are size-fractionated on Sephacryl 500HR columns to remove unlinked adaptors and RNA < 500 base pairs. Parallel column fractions, labeled with <sup>32</sup>P-labeled oligo-dT primer, are used to monitor the size of polyA<sup>+</sup> RNAs to be used for ligations to vector.
  10. Adapted cDNAs are ligated to the BstX1-digested yeast shuttle vector, pYES2.0, and passaged through *E. coli* by electroporation to amplify the library. This pool of transformed *E. coli* is subjected to large scale plasmid isolation and the library is stored as a plasmid DNA pool.

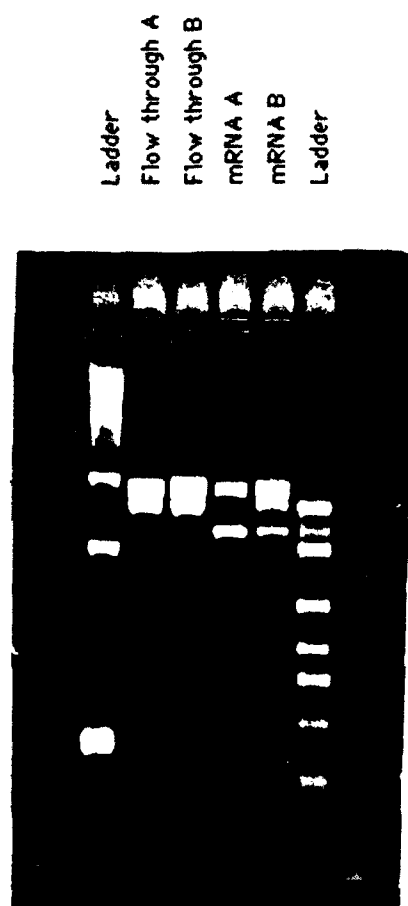
Figures 1 and 2 provide evidence of the success of this protocol.

Figure 1 demonstrates the isolation of poly A<sup>+</sup> RNA from oligo-dT chromatography columns and suggests a reasonable purification of putative mRNAs of various sizes.

Figure 2a and 2b show the size fractionation of cDNAs following isolation of polyA<sup>+</sup> RNA by oligo-dT chromatography and first and second-strand synthesis. Figure 1c shows representative clones from the cDNA library range in size from ~600 to 6000 base pairs.

These studies suggested that (1) we had identified the appropriate experimental parameters which would allow rescue of rad3 mutant strains which exhibited minimal functional gene expression and that (2) we had developed appropriate library construction techniques which would produce cDNAs of a size to permit this rescue. In a series of experiments involving the construction of three independent cDNA libraries and the screening of transformed colonies which represented more than four genome equivalents, a number of apparently transformed yeast RAD3 strains arose. Unfortunately, upon further testing, true transformants were not identified and it was concluded that these strains represented reversions of the original rad3 mutation.

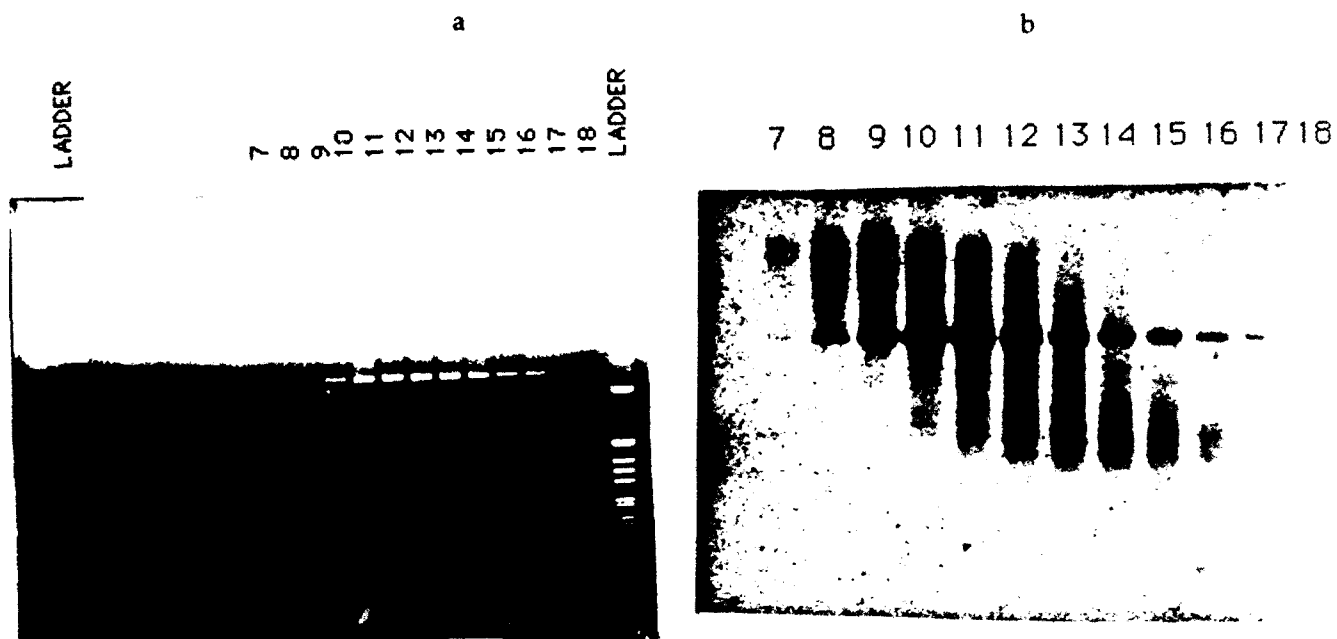
Figure 1. Electrophoretic analysis of cDNA library construction steps.



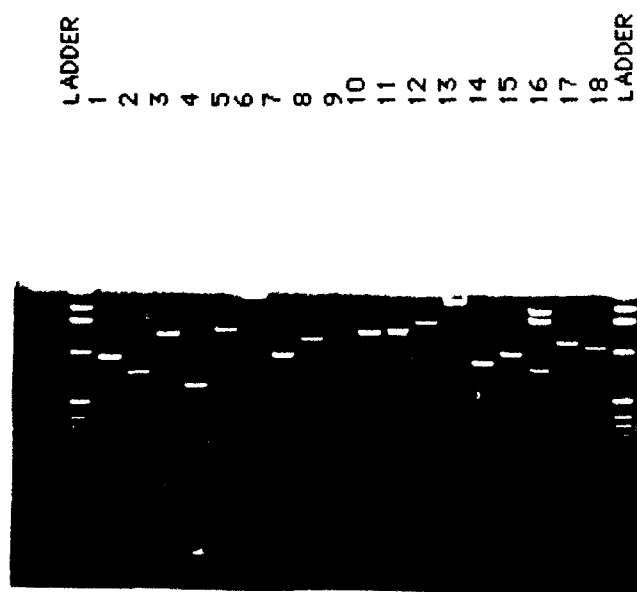
Flow-through and eluant from an oligo-dT column. Lanes numbered from left to right; Lanes 1 and 6 are molecular weight standards; Lanes 2 and 3 represent flow-through volumes, indicating non-polyadenylated RNA, principally ribosomal species; Lanes 4 and 5 represent column eluant of polyadenylated RNA, including a large quantity of an apparently single species of RNA, believed to be mitochondrial in origin (Zimmerman et al., 1980).

Figure 2. a) Sephacryl 500HR column fractions following second-strand cDNA synthesis. Outside lanes indicate molecular weight markers; Inside lanes represent sequential size fractions

b) cDNAs were labeled with  $^{32}\text{P}$  to assay size fractionation. The mitochondrial "contaminant" appears to persist in these fractions. Fractions 7-10 indicate the presence of cDNAs of relatively large size.



c) representative clones isolated from the cDNA library; size range ~600-6000 bp



### *I. Molecular Cloning by PCR-based Sequence Homology*

As an alternative approach, we used the polymerase chain reaction to attempt the isolation of a *Drosophila* cognate of the RAD3 gene. From known sequence information, we were aware that the RAD3 gene had a human homolog, ERCC-2. Using consensus sequence information, two PCR primers, shown below, were synthesized and used as the starting material for PCR reactions.

5' primer (207+21) from ERCC2 sequence:	TCAGGCACCGGGAAGACAGTA
3' primer (1885+21) from ERCC2 sequence:	CATGGCATCGAAGG(T/A)AAGGA

Figure 3 shows the results of an amplification reaction, displayed by electrophoresis and ethidium bromide staining. Lane 1 used cDNAs synthesized from fraction 11 from our oligo-dT columns, described above for the synthesis of our cDNA libraries, as template and lane 2 has molecular weight standards. Interestingly, lane 1 suggests that there is a single DNA species amidst our cDNAs which may have RAD3/ERCC-2 homology. It should be noted that this result was twice independently reproduced, as we wanted to eliminate assay-to-assay variability in the PCR reactions. The DNA from lane 1 has been isolated and cloned and is presently being sequenced.

These data suggest that the PCR approach will be successful in identifying relevant DNA sequences and genes which are critical to the excision repair process in *Drosophila melanogaster*. Unfortunately, the modest seed funding for this project ran out at this point and our request for additional support was denied.

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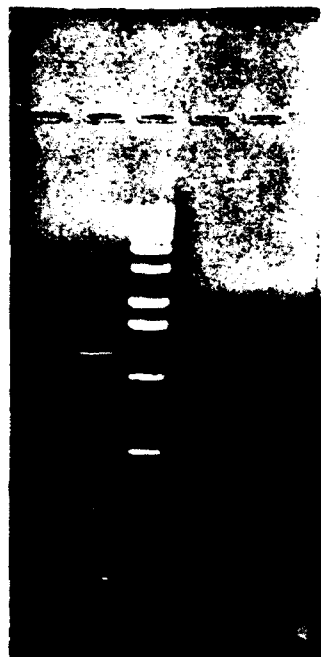
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### **Literature Cited**

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- Weber, C.A., E.P. Salazar, S.A. Stewart and L.H. Thompson (1990) ERCC2: cDNA cloning and molecular characterization of a human nucleotide excision repair gene with high homology to yeast RAD3. *EMBO J.* 9:1437-1447.
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Figure 3. Results of polymerase chain reaction amplifications, using RAD3/ERCC2 consensus primers.



Lane 1 shows a single PCR product, using cDNAs from fraction 11 of our Sephacryl 500HR column, employed in cDNA library constructions for the functional complementation assay, as the DNA amplification source; Lane 2 represents molecular weight markers.

## **Publications**

No publications have yet resulted from this effort. We anticipate that the eventual characterization of the putative RAD3/ERCC-2 *Drosophila* homolog will result in one or more publications in *Mutation Research*, *Cell*, or *Genetics*.

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## **Interactions**

Information was presented at a local Molecular Oncology Program where papers were designed to provide a look at novel strategies for cancer research (Michigan Cancer Foundation, 1991)

## **New Discoveries, etc.**

No major discoveries, inventions, or patents emanated from this project.

## **Other Statements, etc.**

This AFOSR seed grant of just over \$50,000 direct costs provided an opportunity to explore two new avenues by which DNA damage and its repair could be explored in eukaryotes. In one case, the attempt to develop an interspecific functional complementation assay between *Drosophila melanogaster* and *Saccharomyces cerevisiae*, was successful in that many new laboratory techniques were mastered but the goal of recovery of *Drosophila* genes was not realized. In the complementary case, the attempt to identify human repair gene homologs in *Drosophila* by use of the polymerase chain reaction was successful as far as the project was carried. Although no renewed funding was approved, we hope to complete this analysis in time.